Kinetic Analysis of the Transport of Thylakoid Lumenal Proteins in Experiments Using Intact Chloroplasts*

(Received for publication, July 2, 1990)

Cynthia Bauerle†, Jennifer Dorl, and Kenneth Keegstra§

From the Department of Botany, University of Wisconsin, Madison, Wisconsin 53706

The transport of the thylakoid protein plastocyanin has been proposed to occur in two steps: 1) transport across the chloroplastic envelope to the stroma and 2) transport across the thylakoid membrane to the lumen where proteolytic maturation occurs. A partially processed stromal form of plastocyanin has been tentatively identified as a pathway intermediate and as the substrate for the second translocation step (Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K., and Weisbeek, P. (1986) Cell 46, 365–375). In this study, we have examined the transport kinetics of several lumenal proteins under various incubation conditions. Soluble intermediate sized forms were observed in import reactions with plastocyanin precursors from higher plant species and with the precursor of the 33-kDa polypeptide of the oxygen-evolving enhancer complex. The accumulation patterns observed for these soluble intermediate sized forms depended on incubation conditions and could not be consistently explained by a simple model where the intermediate sized form is the substrate for the second step. Thus, it has not been possible to clearly identify the substrate for thylakoid translocation in organello.

Import studies using intact chloroplasts have revealed that the transport of the thylakoid lumen protein plastocyanin (PC) occurs in two steps (1). First, the PC precursor (prPC) is transported across the envelope membrane in a manner similar to that for other imported chloroplast proteins. This transport step requires an internal source of ATP and is directed by the amino-terminal portion of the PC transit peptide, termed the chloroplast-targeting domain (2, 3). The second step of transport is directed by the signal sequence-like thylakoid-targeting domain of the PC transit peptide (4).

Several lines of evidence support the idea that transport to the lumen occurs by direct translocation from the stroma across the thylakoid membrane. First, in import experiments, using wild type *Silene* prPC, a partially processed imported form of PC (iPC) is consistently observed in a soluble fraction containing stromal components, indicating that the transport route proceeds through that compartment (1). Further, prPC deletion mutants that lack a portion of the thylakoid-targeting domain are not properly localized to the lumen but, rather, accumulate in the stromal space (5). Third, a hybrid bipartite targeting signal, consisting of the ferredoxin transit peptide fused to the PC thylakoid-targeting domain, correctly targets PC to the lumen (5). Thus, the chloroplast-targeting domain of the prPC transit peptide can be functionally replaced by a targeting signal that normally directs proteins to the stroma. Finally, the demonstration in the preceding paper (6) that soluble prPC is able to translocate across isolated thylakoid membranes *in vitro* is strong evidence for a transport mechanism in the thylakoid membrane.

Based on the kinetics of iPC accumulation documented in the initial report of *Silene* PC transport in isolated chloroplasts, it was proposed that this stromal intermediate sized form is the translocation substrate for the second transport step (1). Thus, it was proposed that prPC undergoes a proteolytic processing event in the stroma that removes only the chloroplast import domain of the transit peptide. An intermediate sized form of PC that comigrates with iPC on gels is produced by incubating prPC with stromal extracts containing the activity that removes the amino-terminal transit peptide from the precursor of the small subunit of ribulose bisphosphate carboxylase (6, 7). Assuming that iPC is similarly generated in organello, the apparent molecular weight of iPC suggests that processing occurs between residues -20 and -25 of the transit sequence, removing 40–45 residues and leaving the thylakoid transfer domain intact (1).

In the preceding paper (6), we demonstrated that prPC is the substrate for transport across isolated thylakoid membranes (6). Thus, it was of interest to examine which form of PC is transported to the lumen in organello. In order to conclude that iPC is transported to the lumen, it is necessary to demonstrate a precursor-product relationship between iPC and mPC. In this paper, we present the results of a careful examination of plastocyanin transport kinetics in intact chloroplasts. We were interested in defining parameters specific to thylakoid translocation. For instance, finding conditions under which thylakoid transport could be reversibly blocked might enable us to study that step *in organello* by pulse-chase experiments. Although we were not successful in developing a method for specifically inhibiting thylakoid transport, we identified several variables that significantly affected the pattern of iPC accumulation. In particular, under certain conditions the observed iPC accumulation pattern could not be
Transport Pathway of Chloroplastic Lumenal Proteins

MATERIALS AND METHODS

In Vitro Expression of Precursor Proteins—Silene, pea, and Arabidopsis prPCs were synthesized from the expression plasmids pSPPC74, pSPPPC1, and pSPPCCara, respectively (1, 8, 9). Plasmids were linearized by digestion with EcoRI (pSPPC74 and pSPPPC1) or XhoI (pSPPCCara) and transcribed using SP6 RNA polymerase (Bethesda Research Laboratories). Plasmid pUC33, containing the prOEE33 gene, was from Dr. J. Gray, Cambridge University, United Kingdom (10). An EcoRI restriction fragment containing the prOEE33 coding sequence was excised and isolated by gel purification. This fragment was subcloned into pBluescript at the corresponding site in the plolylinker to generate the expression plasmid pBOEE333. Transcription of the BarnHI-linearized plasmid with T3 RNA polymerase (Bethesda Research Laboratories) yielded translatable mRNA. Radiochemically pure precursor proteins were synthesized as described in the preceding paper (6).

Import Conditions—Intact pea chloroplasts were isolated as described (1). Light-driven import reactions were performed in a Gilson respirometer at 25 °C, with a measured light intensity of 250 microEinsteins/m²-s. For reduced temperature experiments, samples were incubated in cover water baths at the specified temperatures in the presence of 1 mM MgATP. In the experiment shown in Fig. 5, equivalent amounts of precursor (μmol of precursor/mg of chlorophyll) were added to chloroplasts that had been preincubated with 1 mM MgATP in the dark for 10 min. Preincubation with ATP increases the stromal ATP concentration, thereby reducing the initial lag phase observed in dark-incubated import reactions and allowing faster import rates (3).

Import/fractionation experiments were performed as described (1). Briefly, intact chloroplasts were reisolated after import and either protease-treated to remove bound prPC (10 μg/ml trypsin/chymotrypsin, 10 min, 0 °C) or osmotically lysed in 50 mM HEPES, pH 8.0, containing 1 mM EDTA at a chlorophyll concentration of 0.1 mg/ml. Chloroplast lysates were separated into thylakoid membrane and soluble fractions by differential centrifugation, and thylakoids were protease-treated to distinguish between bound and luminal PC.

For time course experiments, aliquots of the import reaction mixture were removed at the indicated times and stopped either by 10-fold dilution with ice-cold 1 × import buffer (50 mM HEPES, pH 8.0, 0.5 M sorbitol) followed by chloroplast resolation over 40% (w/v) Percoll (Method I) or by centrifugation through silicon oil (Wacker) into 1 M perchloric acid + 5 mM EDTA as previously described (Method II) (11).

Sample Analysis—Chloroplast samples resolated by Method I were prepared as described in Smeekens et al. (1) and solubilized in 1 × Laemmli gel sample buffer (12). When Method II was used to stop import, samples were prepared in the following way. Sample tubes were cut with a razor blade at the oil/perchloric acid interface, and the acid supernatant was carefully removed from the tip with a pipette. The protein pellet was resuspended in 25 μl of 0.5 M Tris, pH 9.0, and transferred to a 1.5-ml Eppendorf tube by a brief centrifugation of the inverted tube tip placed inside the Eppendorf tube. Pellets were solubilized by adding 25 μl of 2% modified sample buffer (2 × Laemmli sample buffer minus β-mercaptoethanol and bromphenol blue) and heating for 5 min at 60 °C. The protein concentration of these samples was measured using the BCA protein determination kit (Sigma) according to the protocol provided. Before loading on gels, 5 μl of β-mercaptoethanol and 1 μl of 2% bromphenol blue were added and samples were boiled for 2 min.

Samples were analyzed by SDS-PAGE and fluorography, and results were quantified as described (1). To account for the different specific activities (molecules of leucine/molecules of protein) of the processed forms of PC, the data were corrected as described in the previous paper (6).

RESULTS

We have further characterized transport across the thylakoid membrane by examining plastocyanin transport kinetics in organello under various incubation conditions. First, we report the characterization of PC transport with respect to such physical parameters as temperature and light. In a second set of experiments, the import of several different luminal proteins was compared in order to elucidate common aspects of the transport pathway and further characterize targeting signal requirements. The purpose of these studies was to carefully examine the transport kinetics of thylakoid luminal proteins in an attempt to identify the translocation substrate for the second step.

Effect of Light on Transport to the Lumen—The energy requirements for PC transport to the lumen have recently been examined (3). PC transport requires the hydrolysis of ATP in the stroma. In addition, two lines of evidence suggest that a protonmotive force is not involved in PC transport. First, light-driven PC transport is not inhibited by ionophores that disrupt the chloroplastic protonotive force as long as ATP is added to the import reaction. Second, PC transport in the dark can be driven by the addition of exogenous ATP. A protonmotive force is not generated under these conditions since no photosynthetic electron transport occurs in the dark (13). Thus, any differences between PC transport in the dark or the light must be due to some other light-dependent effect.

A comparison of Silene PC import time course experiments, performed either in the light or in the dark, revealed different accumulation patterns for both iPC and mPC. Fig. 1 shows the results of a PC import experiment in which chloroplasts were incubated in the light without added ATP or in the dark in the presence of 1 mM ATP. While rapid accumulation of both iPC and mPC was observed in the light-driven reaction (top panel), a slower rate was observed when import was driven in the dark by the addition of ATP (bottom panel). As described earlier, this rate difference reflects the source of ATP available for driving import (3). That is, the import rate reflects the rapid rise of stromal ATP due to either photophosphorylation (in the light) or the relatively more sluggish accumulation of ATP in the stroma by equilibration across the envelope via the ATP/ADP translocator (in the dark).

Interestingly, although iPC disappeared at later time points

Fig. 1. Import time course of Silene PC in the light and dark. Incubations were carried out at 25 °C in the light (top) or in the dark + 1 mM ATP (bottom). Aliquots were removed at the times indicated in minutes, stopped by Method I, and analyzed by SDS-PAGE and fluorography.
in the light-incubated reaction, it continued to accumulate throughout the time course in the dark. Thus, although iP is in light-incubated reactions appeared to be converted to mP with time, the dark-incubated iP pattern did not fit as well with the interpretation that iP is the intermediate in a two-step pathway. Rather, in the dark, iP accumulation resembles that of mP and is not easily explained by a two-step model in which iP is a pathway intermediate.

An alternative explanation for the light-stimulated reduction of iP over time is that this form of PC is degraded by stromal peptidases. To evaluate the possibility that iP is degraded in the stroma, we performed the following experiment. PC import was carried out for 10 min in the dark to allow accumulation of iP and mP. Chloroplasts were then reisolated and incubated without precursor for 30 min either in the light or in the dark. Samples were removed at various times in order to monitor the amount of iP and mP remaining. Since no further import could occur after reisolation, it was possible to directly monitor the iP already present. If a light-stimulated proteolytic activity is present, then iP should be observed to disappear at a faster rate in the light than in the dark. This is precisely the pattern that was observed (Fig. 2). When the second incubation was conducted in the dark, iP was very stable, with greater than 80% remaining after 30 min (Fig. 2B). In contrast, when the second incubation was conducted in the light, iP disappeared rapidly, dropping to 40% of the original level after only 10 min (Fig. 2B). This drop was not due to conversion of iP to mP since the levels of mP did not increase in either the light or dark (Fig. 2A). In this experiment, it could not be rigorously excluded that mP was being degraded in the lumen at the same rate that it was transported into the lumen, but this explanation seems unlikely as the levels of mP were constant under both light and dark conditions. Thus, these results are consistent with the hypothesis that light-stimulated decreases in the iP pool during import time course experiments are due to increased degradation rather than increased transport across the thylakoid membrane.

Temperature Sensitivity of Transport to the Lumen—To further evaluate the fate of iP, we sought improved ways to investigate the possibility of a precursor-product relationship between iP and mP. While the experiment described above allowed us to follow the decrease in iP over time, the interpretation was made more difficult by the pool of mP already present at the start of the reincubation period. It was thus of interest to find conditions in which thylakoid transport could be specifically and reversibly blocked so that pulse-chase experiments could be performed. Given the distinct lipid compositions of the envelope and thylakoid membranes, we reasoned that translocation across the envelope and thylakoid membranes might be differentially affected by reduced temperature. For instance, if thylakoid translocation were more sensitive, then lowering the temperature should reduce the rate of the second transport step and lead to the accumulation of stromal import intermediates. Subsequent elevation to permissive temperature should allow thylakoid translocation to occur. In this way, we might be able to specifically examine thylakoid transport within intact organelles. Alternatively, if envelope translocation were more affected, then lowering the temperature should lead to an overall reduction of PC import into the organelle.

Time course reactions of Silene prPC import were carried out at 9, 17, and 25 °C. Clearly, transport was slowed by lowering the temperature (Fig. 3). The transport rate at 17 °C was approximately 25% of the rate at 25 °C and was almost completely halted at 9 °C (Panel B). The major accumulated form at reduced temperature was mP, the end product of the transport pathway (Panel A). Low temperature import results with pea prPC were similar (data not shown). Since under these conditions the accumulation of stromal intermediate was not observed, it can be concluded that the rate-limiting step under low temperature conditions was translocation of prPC across the envelope. In similar experiments, the import of the precursor of the small subunit of ribulose bisphosphate carboxylase, a stromal protein, was also slowed by temperature reduction. Since envelope translocation was relatively more sensitive to reduced temperature than thylakoid transport, we were unable to uncouple the transport steps by this method. Neither this approach nor others we have tried allowed us to temporally separate envelope translocation from thylakoid translocation.

The result of the incubation at 25 °C was consistent with that shown in Fig. 1, (bottom panel). That is, initial iP accumulation in the dark for 10 min in the presence of 1 mM ATP. Subsequent to import, intact chloroplasts were resuspended over a 40% Percoll cushion, resuspended in buffer, and reincubated at 25 °C either in the light or in the dark. At the times indicated, samples were removed and stopped by Method II, analyzed by SDS-PAGE/fluorography, and quantified as described under "Materials and Methods." The averaged values from two independent experiments are plotted as a percentage of the amount present at the start of the postincubation period. Panel A, plot of per cent mP remaining during postincubation in the light or dark; Panel B, plot of per cent iP remaining during postincubation in the light or dark.

3 J. Ostrom and K. Keegstra, unpublished results.
the accumulation of a stromal intermediate (9). While PC transit peptides from several species display a bipartite organization, significant primary sequence variation exists that may account for the different import patterns observed (Fig. 4). For instance, failure to observe a stromal intermediate during import experiments with the Arabidopsis precursor may indicate lack of an available processing site in the Arabidopsis PC. Alternatively, different transit peptides may be more efficient at directing transport across the envelope or thylakoid membranes, leading to the accumulation of intermediates in some cases but not in others.

To examine this question, we compared the transport of PC from three higher plant species into pea chloroplasts with respect to accumulation rates of mPC in the lumen and compared the appearance of stromal intermediate sized molecules. Import experiments were performed with prPC from Silene, pea, and Arabidopsis, and the imported products were localized by subsequent fractionation of the organelles into soluble and membrane components. For each species, mPC accumulated in the thylakoid lumen, indicating that proper routing had occurred (Fig. 5, lanes 4–6). For each precursor, soluble intermediate sized molecules were consistently observed in the stromal fraction (Fig. 5, lanes 3). Thus, our results conflict with earlier reports in which no soluble intermediates were observed with the Arabidopsis precursor. In the case of both Arabidopsis and pea PC, two intermediate sized forms were clearly present.

In order to assess more directly the nature of the processing event that produces Silene iPc, differential labeling of prPC was utilized to distinguish amino- and carboxyl-terminal processing events in organello. Import experiments were performed using Silene prPC differentially labeled either near the amino terminus (arginine residues −30 and −42) or at a unique cysteine residue near the carboxyl terminus (residue +84, see Fig. 4). Based on the estimate that iPc has lost 45–50 terminal residues, processing should remove enough of one end of the protein such that either the arginine or cysteine residues should be completely lost. While prPC labeled with either [3H]arginine or [35S]cysteine was visualized by standard fluorography methods, only the cysteine-labeled imported products were detected, suggesting that the amino-terminal arginine residues were lost during import (data not shown). This result supports the interpretation that Silene iPc is generated in organello by proteolytic processing within the amino-terminal transit sequence. It was not possible to examine the intermediate sized forms of pea or Arabidopsis PC by this method due to the lack of unique residues at the amino and carboxyl termini. However, it is most likely that the observed stromal forms were produced by a similar processing event. Estimated amino-terminal processing sites, based on the apparent mobility of the intermediate sized forms in a single percentage gel system, are indicated by arrowheads in the transit sequences shown in Fig. 4.

Comparison of PC Transport Rates—Dark-incubated import time courses were performed with the three prPC species, under conditions in which the initial precursor:chloroplast ratios were similar for each reaction (Fig. 6). For all three precursors, mPC accumulation proceeded at a steady linear rate for approximately 7–10 min, then leveled off. When the quantitative results were presented as “the number of molecules imported/chloroplast”, there was some variability in the extent of import among the different species. Interestingly, when the results with prPC were normalized to the original precursor:chloroplast ratio present at the beginning of the incubation (% imported), all three precursors imported with essentially identical kinetics (data not shown).

Intermediate sized imported molecules (iPCs) accumulated with time for all three PC species (Figs. 5 and 6). In experiments with pea or Arabidopsis prPC, the two intermediate sized forms appeared simultaneously and followed similar accumulation patterns. Consistent with earlier results, no drop in iPc levels was observed at later time points in these dark-incubated import reactions. Moreover, in each case, iPcs were first observed in the time course at a point after which mPC was already present.

Transport Pathway of Pea prOEE33—Import experiments were performed with the precursor of OEE33 (prOEE33). Translation of in vitro-synthesized pBOEE33.3 RNA in a wheat germ extract yielded one major product of 38.5 kDa (Fig. 5, top right panel, lane 1). Upon incubation of prOEE33 with energized chloroplasts, two imported forms accumulated (lane 2). An intermediate sized molecule of 34.6 kDa (OEE33) was soluble in the stroma as evidenced by the localization data (lane 3). The mature sized molecule (mOEE33) was exclusively localized to the thylakoid lumen (lanes 4–6).

An import time course experiment was performed with prOEE33 (Fig. 6, Panel B). Intermediate sized OEE33 was the earliest imported form observed and disappeared from the stroma at later time points. Mature OEE33 appeared later and accumulated throughout the incubation. This pattern differs from that observed with PC import time courses (compare Panels B with A, C, and D). When the normalized time course data for mOEE33 was compared with the results with the different species of PC, the import rate was similar (data not shown). Thus, the transport of a different lumen protein, with a mature “passenger” protein 3 times the size of PC, proceeded with the same rate and efficiency.
A. ALIGNED PC TRANSIT SEQUENCES:

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>Amino Acid Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sil</td>
<td>MATVTSSLAAVTFPSGGLK</td>
<td>(20)</td>
</tr>
<tr>
<td>Pea</td>
<td>MATVTSS-TAIPPSGGLK</td>
<td>(8)</td>
</tr>
<tr>
<td>Ara</td>
<td>MAATIS-AVTTPSFTGLK</td>
<td>(9)</td>
</tr>
<tr>
<td>Bar</td>
<td>MAALS-SAAVSPFPAAAT</td>
<td>(16)</td>
</tr>
</tbody>
</table>

**FIG. 4.** Amino acid sequences of PC transit peptides and mature proteins. Sequences are shown in the single-letter amino acid code. Species abbreviations are: Sil, Silene pratensis (20); Pea, Pisum sativum (8); Ara, Arabidopsis thaliana (9); Bar, Hordeum vulgare (16). Panel A, PC transit sequences. Spaces (−) have been included in order to maximize sequence alignment. Lower case letters indicate a residue within the hydrophobic stretch. Arrowheads mark estimated processing sites within the transit peptides. Panel B, PC mature sequences.

B. ALIGNED PC MATURE SEQUENCES:

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sil</td>
<td>AÆVLGGSDG GLAVPVSSL</td>
</tr>
<tr>
<td>Pea</td>
<td>VEVLLGSDG GLAVPVSESL</td>
</tr>
<tr>
<td>Ara</td>
<td>MVE LLGSDG SLAVPVSESL</td>
</tr>
<tr>
<td>Bar</td>
<td>DVTKISMEP DNLAPGEY S VLTEGTKY</td>
</tr>
<tr>
<td></td>
<td>DAKISMEP DNLAPGETT VS KLDAQKGY</td>
</tr>
<tr>
<td></td>
<td>GKFYCPHAGA GMVGKVTVN</td>
</tr>
<tr>
<td></td>
<td>GKFYCPHAGA GMVGKVTVN</td>
</tr>
</tbody>
</table>

**FIG. 5.** Import of thylakoid lumen proteins and localization of imported products to chloroplast-soluble and membrane fractions. Import and fractionation were performed as described under “Materials and Methods.” Each panel is identified by the name of the precursor used. **TR**, translocation products. **Lane 1**, total chloroplast fraction; **lane 2**, chloroplasts posttreated with protease; **lane 3**, soluble fraction; **lane 4**, thylakoid membranes; **lane 5**, thylakoids posttreated with protease; **lane 6**, sonicated thylakoids, posttreated with protease. The higher molecular weight bands observed in lane 5 of Silene and Arabidopsis PC reactions are proteolytic degradation products of prPC and were not consistently observed in fractionation experiments.

**DISCUSSION**

In earlier studies, kinetic analysis of PC accumulation in light-driven import experiments led to the hypothesis that transport to the lumen occurs in two steps (1). Specifically, the accumulation pattern of iPC suggested that this partially processed form, containing the signal sequence-like domain II, was converted to mPC over time and, thus, was the substrate for transport across the thylakoid membrane. However, there are now several cases in which the transport of a luminal protein has been reported to occur without the accumulation of a soluble intermediate (9, 14, 15). In import experiments using Arabidopsis prPC, or prOEE33 from either wheat or Arabidopsis, targeting to the lumen occurs, but soluble partially processed forms are not observed. Further, as demonstrated in the preceding paper, iPC is not active for thylakoid translocation in vitro. These observations raise questions about the role of stromal processing in the transport of luminal proteins. Thus, it was of interest to investigate which form of PC is transported across the thylakoid membrane in intact chloroplasts.

**Comparison of Silene, Pea, and Arabidopsis PC Transit Peptides**—While PC mature sequences are very highly conserved, significant transit sequence variation exists among the three PC precursors (Fig. 4). We compared the import of three species of PC in order to determine whether differences in transit sequences led to different transport characteristics. The transport rates were similar among the species tested when results were normalized to the initial precursor: chloroplast ratio. Closer examination of the PC transit sequences reveals that most of the sequence variation occurs within the central portion of the transit peptide, whereas the flanking amino- and carboxyl-terminal ends were relatively well conserved (Fig. 4). Given that changes in this region did not correspond to changes in transport efficiency, this region may not be important in the proper functions of the PC transit peptide. Interestingly, the PC transit sequence from barley lacks most of this region in comparison with other PC transit sequences, suggesting that it may be dispensable (Fig. 4, see also Ref. 16).

Intermediate sized partially processed forms were observed in import experiments with all of the PC species as well as the pea OEE33 precursor. While only one intermediate sized
form was observed for Silene PC, two different forms were seen in time courses of pea and Arabidopsis PC. These intermediate sized forms comigrated with the products of in vitro processing with stromal extracts (see accompanying paper). In the current study, we could not strictly exclude the possibility that these stromal forms were products of an aberrant proteolytic event. However, since amino-terminal processing was demonstrated for the Silene PC, it is likely that pea and Arabidopsis iPC forms were generated by a similar activity. Further, amino-terminal processing has been confirmed by microsequence analysis of the in vitro-generated OEE33 intermediate.3

Involvement of Intermediate Processing in the Transport of Lumenal Proteins—In the kinetic studies described above, the iPC accumulation pattern differed, depending on the incubation conditions. When import reactions were carried out in the dark, iPC accumulated slowly, appearing after mPC was observed in the lumen, and continued to accumulate in the stroma for up to 30 min (Fig. 1, bottom panel). This pattern was observed in import experiments using three different species of prPC (Fig. 6, A, C, and D). Furthermore, the lag between the initial appearance of mPC and the first observation of iPC in the dark was exaggerated at reduced temperatures (Fig. 3A). These results contrast with data from light-driven experiments in which iPC accumulated early and appeared to chase with time (Fig. 1, top panel). It is difficult to interpret these combined results in terms of a simple two-step pathway in which iPC is the substrate for thylakoid transport. In particular, the appearance of mPC prior to iPC accumulation under certain conditions calls into question the requirement for intermediate processing in the PC transport pathway.

In contrast, the import of pea prOEE33 in the dark proceeded with the accumulation of a partially processed form that behaved kinetically like a pathway intermediate (Figs. 5 and 6B). A similar intermediate sized molecule has been observed in import studies using the spinach precursor, although the accumulation pattern appears to be more similar to that observed in PC transport reactions (17). However, it has been reported that no stromal intermediates are observed in import reactions with prOEE33 from wheat or Arabidopsis (14, 15). These combined results suggest that, for this precursor as well, intermediate formation may not be a required step in transport to the lumen.

In light of these results, partial transit peptide processing, as visualized by the accumulation of stromal intermediates, may not be a required step in the transport pathway of thylakoid lumen proteins. Thus, although iPCs are consistently observed in PC import reactions, their role in the transport pathway is unclear. Fig. 7 summarizes the possible transport and processing steps for lumenal proteins. All arrows without question marks indicate events that have been demonstrated to occur (see text). The fate of iPC is uncertain, as indicated by the question mark adjacent to the two arrows leading from stromal iPC.

Fig. 6. Import time courses of thylakoid luminal proteins. Time course experiments were performed at 25 °C, as described in the legend to Fig. 2, except that time point samples were stopped by Method II. Samples were prepared and quantified as described under Materials and Methods. Panel A, pea PC; Panel B, pea OEE33; Panel C, Silene PC; Panel D, Arabidopsis PC. The number of precursor molecules presented at the start of import was: pea PC, 787 mol/chloroplast; pea OEE33, 413 mol/chloroplast; Silene PC, 1384 mol/chloroplast; Arabidopsis PC, 1190 mol/chloroplast. Legends are included in each graph.

Fig. 7. Summary of the transport pathway of thylakoid luminal proteins in intact chloroplasts. Shaded arrows indicate membrane transport events and line arrows indicate proteolytic processing events. All steps without question marks have been demonstrated to occur (see text). The fate of iPC is uncertain, as indicated by the question mark adjacent to the two arrows leading from stromal iPC.

3 C. Robinson, personal communication.
intermediates seen in time course experiments. These intermediates may be slowly transported to the lumen and converted to mPC. Alternatively, these accumulated molecules may be translocation-incompetent. The time-dependent decrease in iPC seen in some time course experiments might represent instead the rate at which these molecules are degraded by other stromal proteases.

In the preceding paper (6), we demonstrated that the active substrate for thylakoid translocation in vitro is prPC. Further, we concluded that iPC is inactive in this isolated thylakoid system. This result may be specific for the in vitro system in which a required soluble factor may be present in limiting amounts. However, it is not possible to exclude the possibility that in organello, prPC may be competent for transport across the thylakoid membrane. Consistent with this idea, the thylakoid peptidase responsible for mature processing can accept either iPC or prPC as substrates in vitro (18). We have not observed stromal prPC in import experiments with intact chloroplasts. However, it is possible that imported prPC is either rapidly transported to the lumen or converted by processing to iPC so that we were unable to observe it. The transport of Arabidopsis PC to the lumen was earlier reported to proceed without the appearance of soluble intermediates (9). In contrast, we consistently observed the accumulation of two soluble intermediate sized molecules in import experiments using the Arabidopsis precursor (Figs. 5 and 6D). The most likely explanation for this derives from differences in import conditions, specifically the light conditions used by Vorst et al. (9) versus the dark conditions employed here. Alternatively, the conflicting results may be explained if the precursor:chloroplast ratio in the experiments shown here was higher than in those reported by Vorst et al. That is, higher rates of PC transport into chloroplasts as supported by higher precursor:chloroplast ratios would result in the observed accumulation of stromal intermediates.

The concept of different rates for the two steps may also be used to explain the observation of stromal intermediates in recent experiments using lumen-targeted chimeric precursors. Two groups have used the transit peptides from wheat and Arabidopsis OEE33 to route foreign passenger proteins to the lumen (15, 19). As stated above, stromal intermediates were not visualized in import experiments with the corresponding wild type prOEE33s. In contrast, intermediate sized forms were clearly observed in import experiments using these chimeric constructs. The observation of stromal intermediate sized forms demonstrates that these transit peptides are able to undergo processing; that is, the sequences contain sites that are recognized by a stromal enzyme. Perhaps although these chimeric proteins import normally into chloroplasts, they are less efficiently translocated into thylakoids than is wild type OEE33, and stromal forms accumulate. That is, stromal intermediates are observed when translocation across the envelope proceeds more efficiently than translocation across the thylakoid membrane.

We have examined the kinetics of PC transport into isolated pea chloroplasts under various conditions in an attempt to identify the translocation intermediate in the two-step pathway. An attempt to specifically block thylakoid transport by performing incubations under reduced temperature conditions was not successful. Similarly, it was not possible to perform PC import experiments under conditions in which stromal processing to iPC was blocked (data not shown). Thus, we were not able to demonstrate a precursor-product relationship between iPC and mPC by this approach.

Our current results, combined with recent reports from other laboratories, call into question the role of the stromal processing in the transport pathway of lumenal proteins. Specifically, it is not possible to exclude that newly imported precursors may be transported across the thylakoid membrane prior to proteolytic processing. It is also not clear whether stromal intermediates are active for transport in organello or translocation-incompetent, as they are in vitro. Clear identification of the substrate for thylakoid transport in intact chloroplasts may only be accomplished by pulse-chase experiments in which the pathway intermediate is allowed to accumulate and then is chased to mPC. Identification of the transport intermediate should increase our understanding of the conformational requirements for thylakoid translocation.

Acknowledgments—We would like to thank Dr. P. Weisbeek and Dr. J. Gray for generous gifts of expression plasmids for prPC and prOEE33 proteins. C. B. would like to acknowledge Kathy Archer, Hsou-min Li, Laura Olsen, and other members of the Keegstra lab for helpful comments and discussion during the preparation of these manuscripts.

REFERENCES